Review

Cell PRESS

Protective immunity to pre-erythrocytic stage malaria

Robert J. Schwenk[1,2] and Thomas L. Richie[1,3]

[1] US Military Malaria Vaccine Program
[2] Walter Reed Army Institute of Research, Division of Malaria Vaccine Development, 503 Robert Grant Avenue, Silver Spring, MD 20910, USA
[3] Naval Medical Research Center, Infectious Disease Directorate, 503 Robert Grant Avenue, Silver Spring, MD 90910, USA

The development of a vaccine against malaria is a major research priority given the burden of disease, death and economic loss inflicted upon the tropical world by this parasite. Despite decades of effort, however, a vaccine remains elusive. The best candidate is a subunit vaccine termed RTS,S but this provides only partial protection against clinical disease. This review examines what is known about protective immunity against pre-erythrocytic stage malaria by considering the humoral and T cell-mediated immune responses that are induced by attenuated sporozoites and by the RTS,S vaccine. On the basis of these observations a set of research priorities are defined that are crucial for the development of a vaccine capable of inducing long-lasting and high-grade protection against malaria.

The scope of the problem

Malaria is caused by the protozoan genus *Plasmodium* and is responsible for 700 000 to 1 000 000 deaths per year in tropical regions of the world, most severely affecting children in sub-Saharan Africa [1]. Naturally acquired immunity to malaria is slow to develop, non-sterilizing and short-lived in the absence of continuous exposure. The parasite rapidly develops resistance to anti-malarial drugs and treatment is often followed by reinfection [2]. Hence, there is an urgent need to develop a malaria vaccine.

This task has proven difficult: after decades of effort, the only candidate vaccine approaching licensure is RTS,S [3], a virus-like particle, based on the dominant surface protein [designated the circumsporozoite protein (CS protein)] of the *Plasmodium falciparum* (*Pf*) sporozoite. RTS,S has the potential to provide a significant health benefit if the reductions in clinical illness seen in early field trials are confirmed in the Phase III trial currently underway in nine countries across sub-Saharan Africa [3]. However, RTS,S does not appear potent enough to prevent infection completely in the majority of vaccine recipients, inducing only partial immunity. To design a vaccine providing life-long, sterile protection, it will be important to understand better the interface of the parasite with the human immune system, including its immune-evasion mechanisms. This objective is especially relevant for the early (pre-erythrocytic) sporozoite and liver stages of the infection where the induction of sterilizing immunity would completely prevent blood stage malaria and thus both clinical illness and transmission.

This review highlights what is known about the immunological responses to the pre-erythrocytic stages of the parasite, focusing on two models of protection: immunization with attenuated sporozoites and immunization with the CS protein. It then identifies key research questions to guide the development of a highly effective pre-erythrocytic stage vaccine.

The sporozoite

When an infected female *Anopheles* mosquito bites, it injects several hundred sporozoites into the cutaneous tissue. Over the next minutes to hours some of the sporozoites leave the injection site and enter blood vessels where they are carried rapidly to the liver, while others remain in the skin or travel to regional lymph nodes or to the spleen. On reaching the liver, sporozoites cross the endothelium, in some cases passing through Kupffer cells (KC), and then traverse several hepatocytes before entering a terminal hepatocyte via an invagination process, leading to the formation of a parasitophorous vacuole in which each sporozoite differentiates into liver stage and finally blood stage parasites [4–6] (Figure 1). As the sporozoite passes through a hepatocyte it can cause cell wounding [4] and in some instances necrosis, although whether or not this constitutes a danger signal for the immune system is unclear. The liver stage of infection appears otherwise to be relatively immunologically silent, possibly due to the minor antigen (Ag) load, the immune-tolerant nature of the liver, and/or the immune-regulatory mechanisms initiated during sporozoite passage through the skin [7].

The attenuated sporozoite human protection model

In 1973 it was demonstrated that humans subjected to approximately 1000 to 2000 irradiated and *Pf* sporozoite-infected mosquito bites, followed by primary challenge within 10 weeks after immunization, were protected and the protection lasted for up to 10 months against sporozoite re-challenge [8,9]. This work followed studies where immunization of animals with radiation-attenuated sporozoites was similarly highly protective [10,11]. Recently, immunization of rodents with genetically attenuated sporozoites (GAS) also afforded high-grade (>90%) protection [12]. Given the powerful immunity induced by attenuated

Corresponding author: Schwenk, R.J. (robert.schwenk@amedd.army.mil).

  doi:10.1016/j.pt.2011.02.002 Trends in Parasitology xx (2011) 1–9

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
2011		**00-00-2011 to 00-00-2011**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Protective Immunity To Pre-erythrocytic Stagemalaria	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
Walter Reed Army Institute of Research,Division of Malaria Vaccine Development,Silver Spring,MD,20910	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES
Trends in Parasitology,No.x,(2011)1-9

14. ABSTRACT
The development of a vaccine against malaria is a major research priority given the burden of disease ,death and economic loss inflicted upon the tropical world by this parasite. Despite decades of effort, however, a vaccine remains elusive. The best candidate is a subunit vaccine termed RTS,S but this provides only partial protection against clinical disease.This review examines what is known about protective immunity against pre-erythro-cytic stage malaria by considering the humoral and T cell-mediated immune responses that are induced by attenuated sporozoitesandbytheRTS,Svaccine.On the basis of these observations a se tof research priorities are defined that are crucial for the development of a vaccine capableofinducinglong-lastingandhigh-grade protection against malaria.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**9**	

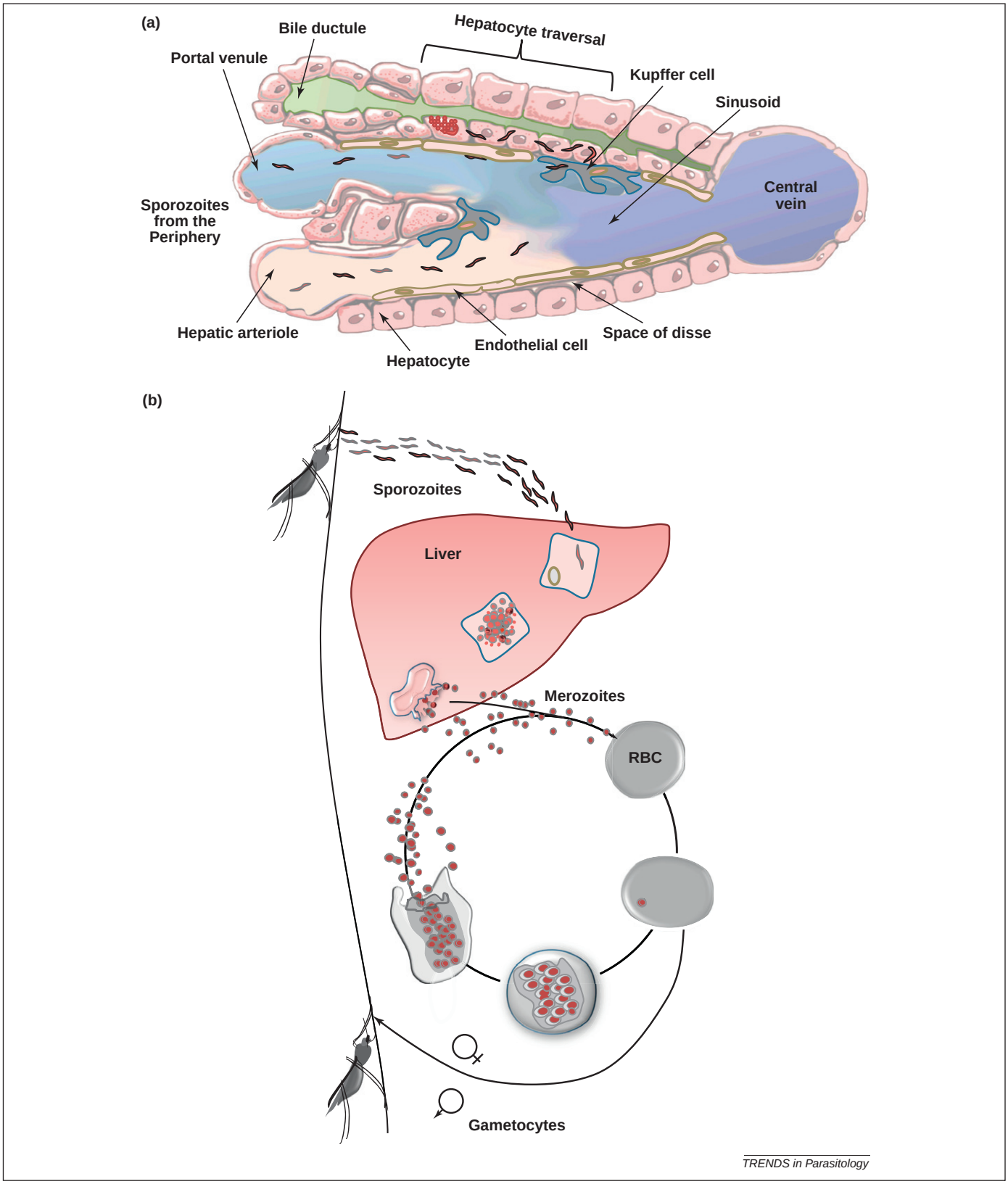

Figure 1. The *Plasmodium* life cycle. **(a)** Sporozoites enter the liver lobule either via the portal venule or the hepatic artery, and arrest by binding to the sinusoidal cell layer. The parasites glide along the sinusoid, sometimes moving against the flow of blood, until they encounter a Kupffer cell. After a pause, they slowly pass through the Kupffer cell and cross the space of Disse beyond it. Once inside the liver parenchyma, the parasites increase their velocity and migrate for many minutes through several hepatocytes, before they eventually come to rest in a terminal hepatocyte to initiate development (panel reproduced with permission from [5]). **(b)** An infected female *Anopheles* injects up to several hundred sporozoites into the cutaneous tissue while feeding. Some of the sporozoites leave the injection site and enter blood vessels where they are carried to the liver. Some of the sporozoites invade hepatocytes (panel **(a)**) where they undergo asexual multiplication (schizogony) and develop into thousands of merozoites. The merozoites exit the infected hepatocyte in membrane-bound vesicles (merosomes or extrusomes) which, following rupture, enable the merozoites to rapidly invade red blood cells thereby initiating blood-stage malaria. During the blood stage, formation of gametocytes also occurs and these are ingested by a feeding mosquito to initiate the next cycle.

sporozoites, much effort has been expended to identify the underlying protective mechanisms, including the antigenic targets.

Radiation-attenuated sporozoites (RAS), as do native sporozoites, invade hepatocytes and form a parasitophorous vacuole, but their development is arrested at the early liver stages [13], creating a repository of early liver stage Ags which could serve to induce and maintain protracted immunity. The relatively persistent Ag load is one factor that distinguishes immunization with attenuated sporozoites from exposure to intact sporozoites under natural conditions. The CS protein and probably other parasite Ags cross the vacuolar membrane, possibly via a 'gatekeeper complex' [14], to enter the cytoplasm where they undergo processing and subsequent presentation on the hepatocyte surface. It has been demonstrated that sporozoite-infected hepatocytes can process and present *Plasmodium berghei* (*Pb*) CS-protein epitopes to T cells [15], although it is also possible that sporozoites and liver-stage Ags egress from the cell to be ingested and processed by dendritic and other Ag-presenting cells. Likewise, some of the RAS entering the liver are directly taken up by dendritic cells (DC) for processing and presentation to T cells. It is probable that multiple antigens are involved in protection, in as much as CS protein contributes to but is not required for protection, because mice tolerized to CS protein can still be protected by RAS [16].

The RTS,S human protection model

The surface of the sporozoite is densely packed with protein. The most prominent is the CS protein which is shed as the parasite undergoes motility [17] and cell traversal [18]. The *Pf* CS protein has N and C terminal regions which flank a central repeat region consisting of 38 to 40 copies of the B cell epitope NANP, preceded by a minor central repeat sequence of alternating NVDP and NANP. Several CD4 and CD8 T cell epitopes have been mapped to the C terminus of the CS protein, and some of these are universal epitopes [19–22]. Currently, there are also extensive efforts to identify additional sporozoite proteins [23–25].

The relatively potent immunogenicity and prominent location on the sporozoite surface have made the CS protein a leading candidate Ag for malaria vaccines. Following the demonstration that CS-protein-based vaccines can induce protection in animals, the RTS,S vaccine was developed to extend this protection to humans [3,26]. RTS,S consists of the central repeat region and the C terminus of the *Pf* CS protein fused to the surface Ag of hepatitis B virus; this is coexpressed with free surface Ag to form an hepatitis B surface (HBs) Ag-like particle. RTS,S has been formulated in different adjuvants including ASO2A, an oil-in-water emulsion containing QS-21 (a derivative of the saponin Quil A) and mono-phosphoryl lipid A, and ASO1B, which is a liposome formulation. The RTS,S vaccine confers sterile immunity to 40–50% of malaria-naïve subjects against a primary sporozoite challenge, and 40–50% of subjects protected against the primary challenge are also protected against rechallenge 5 months later [27]. The Ab- and cell-mediated immune responses to RTS,S have been characterized extensively in both protected and non-protected human volunteers [27,28].

Ab-mediated immunity

There is evidence that humoral immunity contributes to protection against pre-erythrocytic stage malaria. Passive transfer of monoclonal Ab specific for the central repeat regions of *Pb* and *P. yoelii* (*Py*) CS protein protects mice against sporozoite challenge [29,30]. Immune serum from sporozoite-immunized humans can also block the invasion of *Pf* sporozoites into hepatocytes [31]. In addition, the RTS,S vaccine induces high titers of CS-protein-specific Abs that correlate with protection against infectious challenge. The mechanism underlying Ab-mediated protection could involve inhibition of hepatocyte invasion or opsonization of sporozoites for uptake by macrophages and DC. Recent studies [32] have also shown that sporozoites injected into the dermis of immunized mice are immobilized and become degraded within hours. Moreover, mosquitoes introduce fewer sporozoites into immunized as compared to non-immunized mice, presumably due to the formation of Ag–Ab complexes in the proboscis [32]. Consequently, RTS,S-induced Abs probably also inhibit sporozoite motility.

Because sporozoites invade hepatocytes soon after injection, sufficiently high titers of sporozoite-specific Abs must be present at the time of injection for Abs to confer protection. This implies a need for sustained Ab production by long-lived plasma cells (PC) (Figure 2). The activation of these cells appears to involve Ags expressing both a repetitive epitope to mediate high B cell receptor cross-linking and T cell epitopes to induce the activation of follicular T-helper (T_{FH}) cells [33]. Particular attenuated virus vaccines meet these criteria and can induce Ab responses that persist for decades without apparent re-boosting [34,35]. Long-term storage of Ag–Ab–complement complexes on follicular DC [36] could also be required.

For the RTS,S vaccine the majority of non-protected volunteers make much weaker CS-protein-specific Ab responses than protected volunteers. In addition, CS-protein-specific Ab titers fall substantially with time after the 3rd dose, even when accompanied by natural boosting [37]. Hence, mechanisms that sustain high Ab titers would probably also improve long-term vaccine efficacy. In general, higher primary Ab titers and longer intervals between boosting tend to favor longer-lived Ab responses, and it would therefore be worth evaluating at 0, 1, and 6 months instead of in the standard 0, 1, and 2 months immunization schedule. There is also evidence [38] that high avidity Abs are more protective than low-avidity Abs, thus methods to increase Ab avidity would probably improve RTS,S-induced protection.

CD4 T cell immunity

There is evidence that CD4 T cells also protect against the pre-erythrocytic stages of malaria. CD4 T cells specific for a *Py* CS-protein epitope can both eliminate infected hepatocytes *in vitro* and adoptively transfer protection *in vivo* [39]. As mentioned, the RTS,S vaccine induces high titers of CS-protein-specific IgG Abs, and this probably depends on the activation of CS-protein-specific CD4 T_{FH} cells. RTS,S-primed CD4 T cells can also be recalled with CS-protein peptides to produce IFN-γ [27], a known inhibitor of intracellular stages of the parasite [40]. Moreover, the

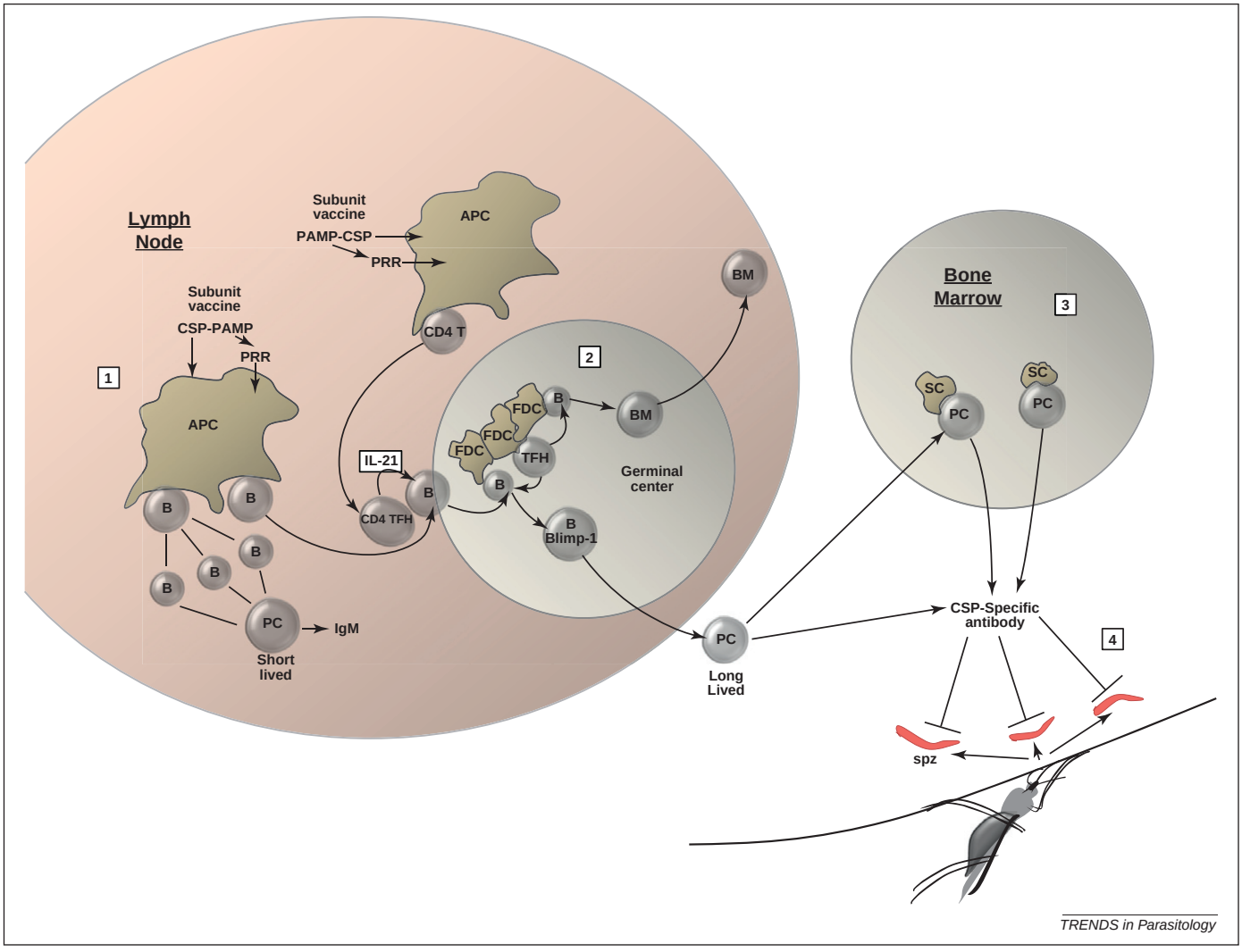

Figure 2. Induction of humoral immunity. **Region 1:** a subunit vaccine – in this case the circumsporozoite (CS) protein coupled to a pathogen-associated molecular pattern (PAMP) – is taken up into an endosome by an Ag-presenting cell (APC). The PAMP interacts with a pattern-recognition receptor (PRR), either on the cell surface or within the endosome, and this stimulates maturation of the APC (Figure 3 legend). Following digestion of the protein within the endosome and binding of the resulting peptide fragments to the binding-grooves of class II major histocompatibility molecules, recognition of CS-protein peptide plus class II molecule on the APC surface by an Ag-specific $CD4^+$ T cell induces upregulation of CXCR5 and differentiation of the $CD4^+$ T cell into a follicular T-helper cell (T_{FH}). B cell activation also occurs, causing some B cells to differentiate into short-lived plasma cells (PC) and others to migrate to the interface between the T cell region and the B cell follicle. **Region 2:** further interaction of the B cells with the T_{FH} cells initiates the germinal center reaction. The B cells start to proliferate, form long-lived complexes with a network of follicular DC (FDC), and eventually differentiate into memory B (B_M) cells or into B-lymphocyte-induced maturation protein (Blimp-1) positive B cells and then into long-lived PC. **Region 3:** the PCs migrate to the bone marrow where they reside in association with stromal cells and secrete IgG Ab for very long periods of time. **Region 4:** this CS-protein-specific Ab can immobilize sporozoites immediately following injection by the mosquito and hence can confer protection against infection.

frequency of the RTS,S-induced IFN-γ^+ CD4 T cells was higher in protected than in non-protected volunteers [27].

Studies of other pathogens in both mice [41] and humans [42] indicate that multifunctional CD4 T cells simultaneously producing IL-2, IFN-γ and TNF-α more strongly associate with protection than bi- or mono-functional CD4 T cells. This could be due to the capacity of IL-2 to promote lymphoproliferation and hence maintain the lineage and the capacity of IFN-γ and TNF-α to act synergistically. In addition, the intensity of IFN-γ secretion from multifunctional cells appears to be greater than that of terminal effector cells. Seder *et al*. [43] have proposed a model in which CD4 T cells progressively gain and then eventually lose effector function as they proceed along the following linear differentiation pathway: IL-2^+ TNF-α^+ cells → IL-2^+ TNF-α^+ IFN-γ^+ cells → [TNF-α^+ IFN-γ^+] or [IL-2^+ IFN-γ^+] cells → IFN-γ^+-only terminal effector cells. They propose that an ideal vaccine will be strong enough to drive CD4 T cells to differentiate into IL-2^+ TNF-α^+ IFN-γ^+ triple producers (referred to as the 'sweet spot'), but not so strong as to further differentiate them into terminal effectors.

The CD4 T cell lineage also includes regulatory T (T_{reg}) cells which are generally controlled by the transcription factor FOXP3 (reviewed in [44]) and which limit excessive inflammation and inhibit sterilizing immunity to pathogens. For example, T_{reg} cells prevent effector T cells from completely eliminating *Leishmania* parasites from the site of infection, and the persistence of residual parasites contributes to the maintenance of long-term immunity [45]. There are several reports that T_{reg} cells control immune responses to blood stage malaria [46,47] and, although there is no direct evidence, they could also regulate responses to the pre-erythrocytic stages. It is clear that

future immunization strategies might have to consider whether a vaccine activates T_{reg} cells in addition to effector and memory T cells and, if this is the case, identify a method to counteract the effects of T_{reg} cells such as the coadministration of an immunomodulatory drug [46].

CD8 T cell immunity

Although Abs and CD4 T cells contribute to protection, CD8 T cells appear to be the *sine qua non* effectors that confer sterile immunity in some strains of attenuated sporozoite-immunized mice. Thus, for example, depletion of $CD8^+$ cells from RAS-immunized BALB/c mice before challenge abrogates protection. In addition, CD8 transgenic T cells specific for *Py* CS protein can secrete IFN-γ in response to and protect mice against sporozoite challenge. Recently it has been demonstrated that GAS immunization of mice also activates CD8 T cells that are required for protection [48] and which can effect contact-dependent killing of *Py*-infected hepatocytes [49].

CD8 T cells specific for *Pf* CS protein have also been found in the blood of both RAS-immunized volunteers and naturally exposed subjects [21,50]. In addition, investigators working at the University of Oxford have explored the use of vaccines encoding the pre-erythrocytic stage thrombospondin-related adhesion protein Ag (TRAP) fused to a string of linked T- and B-cell epitopes (ME-TRAP), and showed that priming humans with a construct of chimpanzee (Ch) adenovirus 63 encoding ME-TRAP (AdCh63–ME-TRAP), followed by a boost with a modified vaccinia Ankara (MVA) construct MVA–ME-TRAP, induced strong CD8 γ-interferon-secreting T cell responses and the associated efficacy data has been submitted for publication (Ewer *et al.*, submitted, Adrian Hill, personal communication).

There is evidence that some sporozoites migrate from the dermis to the draining lymph node where they activate CS-protein-specific CD8 T cells [51]. These CD8 T cells subsequently travel to the liver where they could contribute to pre-erythrocytic stage immunity. It is more likely, however, that attenuated sporozoites also induce CD8 T cells that are specific for the liver-stage Ags developing only in infected hepatocytes.

Attenuated sporozoites activate CD8 T cells, whereas native sporozoites do not, and this could be related to the former causing infected hepatocytes to undergo apoptosis [52,53]. The apoptotic cells are probably taken up by intrahepatic $CD8^+$ DC for cross-presentation of liver-stage Ags to CD8 T cells [54]. This mechanism, however, has been called into question [55], in part because native sporozoites can also induce sterile immunity in humans when administered with chloroquine to prevent blood stage infection [56], although there is the additional proviso that immunity in this case could have been directed primarily against very early blood stages that exit the liver. Protection, in this case, appears to be mediated by multi-functional effector memory T (T_{EM}) cells, and chloroquine could have potentiated the activation of these cells [57].

In general, acute infection of humans or mice with a pathogen causes CD8 T cells to undergo rapid expansion to generate a large population of effector cells. Upon pathogen clearance, the cells undergo rapid contraction when most of the cells die by apoptosis. However, 5–10% of the effector cells survive and form long-lived memory CD8 T cells. The extent of contraction, and the maintenance of memory cells, appear to depend on several γ_c cytokines including IL-7, IL-15 and IL-21. Immunization of mice with RAS or GAS results in the accumulation in the liver of both $CD44^+$ $CD62L^-$ $CD122^-$ $CD8^+$ effector/effector memory T($T_{E/EM}$) cells and $CD44^+$ $CD62L^+$ $CD122^+$ $CD8^+$ central memory T (T_{CM}) cells [58,59]. Surprisingly, the CD8 $T_{E/EM}$ cells do not undergo contraction after the last boost/immunization or after sporozoite challenge, even after a prolonged period of time. It is conceivable that the lack of contraction is due to chronic Ag stimulation, and this would be consistent with RAS immunization generating a persisting Ag depot. It is not clear whether protection against sporozoite challenge requires the protracted presence of CD8 T_E cells that could respond immediately to infection. If, by contrast, protection depends on CD8 T_{CM} cells that can only be recruited from a draining lymph node, there could be insufficient time for these cells to recognize new parasite Ag, multiply to high numbers, and migrate to the liver to kill the parasite. Conversely, if CD8 T_{CM} cells are maintained in the liver, temporal constraints might not apply. One of the key questions for the development of a malaria subunit vaccine is, therefore, whether protection against sporozoite challenge requires persisting CD8 T_E cells or CD8 T_{CM} cells and, if the former, whether the maintenance of these cells depends on a persisting depot of Ag. It is worth noting that primaquine treatment to eliminate persisting liver-stage Ag before the development of long-lived memory cells abolished protection in both RAS [60] and GAS [61]-immunized animals.

Valuable data regarding the role of central versus effector memory CD8 T cells have been provided by investigators at the University of Oxford. Volunteers were immunized with ME-TRAP; DNA and fowlpox constructs were used for priming, and an MVA construct provided the boost. In some cases, vaccine-induced reduction in liver-stage burden correlated more closely with long-term (cells cultured for 9 d) than with short-term (cells cultured for 18 h) responses as measured by enzyme-linked immunospot (ELISPOT) assay, indicating that T_{CM} cells present at the time of sporozoite challenge could make a greater contribution to protection than T_{EM} cells [62,63]. A principal hindrance to answering these questions in the RAS model is our lack of knowledge of the Ags expressed by the liver stage of the parasite. Consequently, extensive efforts employing both genomics and proteomics are being directed to discovering relevant liver stage Ags [23–25]. For example, 16 novel *Pf* proteins have been recently identified which peripheral blood mononuclear cells (PBMC) from RAS-immunized volunteers recognized more frequently than 'traditional' *Pf* Ags such as the CS protein and exported protein-1 (EXP-1) [64]. Moreover, two of these 16 Ags elicited higher responses from protected versus non-protected volunteers.

Once the mechanisms that enable attenuated sporozoites to induce sterile CD8 T-cell-mediated immunity are understood, attempts could be made to trigger similar

immune mechanisms by a subunit vaccine. The initiation of the appropriate activation pathways will probably occur in the lymph node draining the vaccination site. Figure 3 illustrates the cellular interactions that lead to the activation of short-lived effector CD8 T cells (SLECs), memory potential effector CD8 T cells (MPECs) and memory CD8 T cells, as well as the migration of these cells from the lymph node to the liver to mediate their effector function.

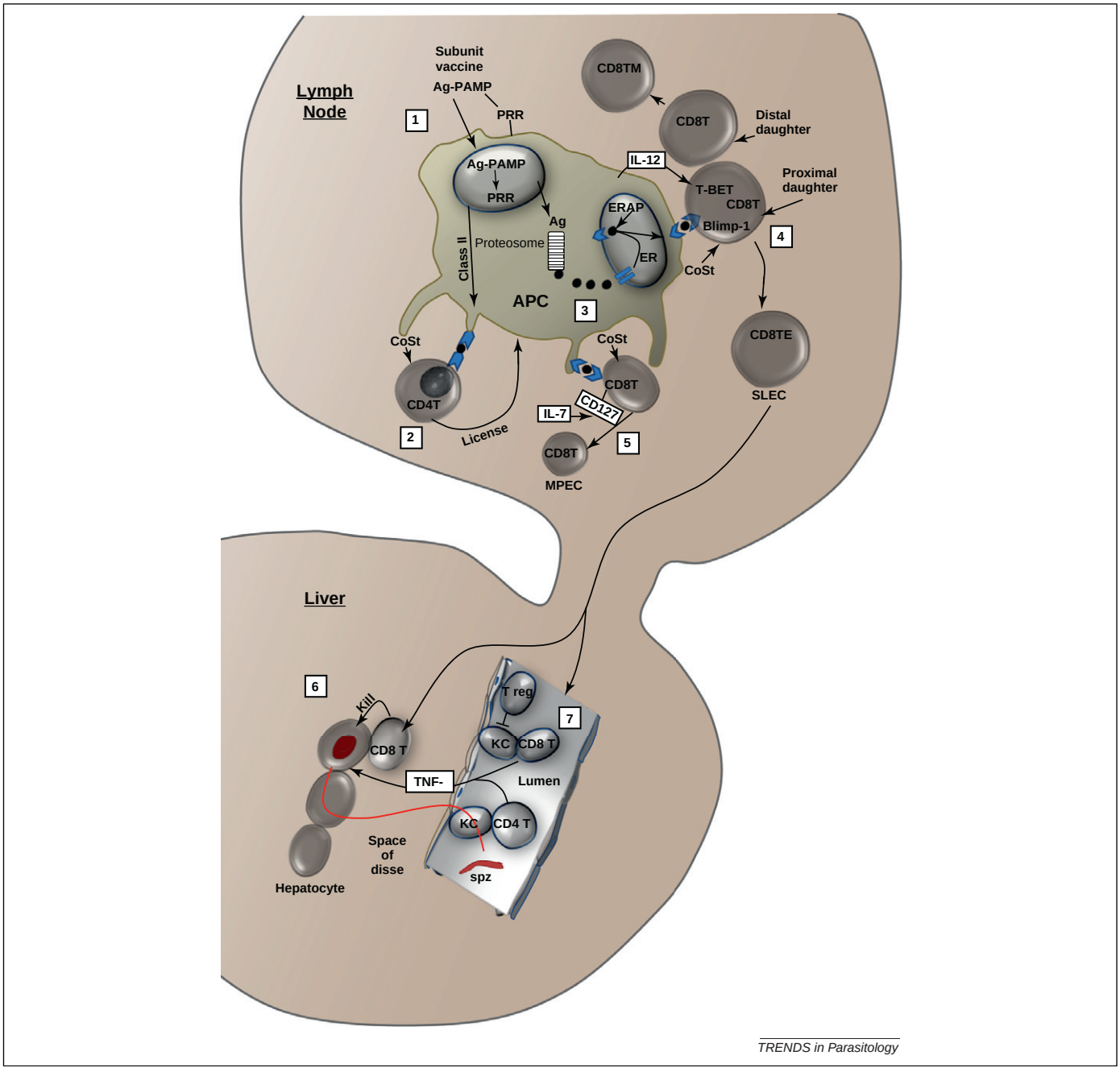

Figure 3. Induction of T cell mediated immunity. **Region 1:** a subunit vaccine – in this case a liver-stage antigen (Ag) coupled to a pathogen-associated molecular pattern (PAMP) – is taken up into an endosome by an Ag-presenting cell (APC). The PAMP interacts with a pattern-recognition receptor (PRR), either on the cell surface or within the endosome, and this stimulates the maturation of the APC. **Region 2:** Ag within early endosomes is degraded and binds to nascent class II molecules for transport to the cell surface. Recognition of the peptide class II complex, followed by co-stimulation (Co St), induces the activation of Ag-specific CD4 T cells and these cells license the APC and enable it to activate CD8 T cells. **Region 3:** some Ag is translocated out of the endosome into the cytoplasm where it is degraded into peptides by the proteosome. The peptides are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) where they are bound to nascent class I molecules and then trimmed to size by the ER aminopeptidase (ERAP) enzymes. The peptide class I complexes are then transported to the cell surface. **Region 4:** the peptide class I complexes are recognized by Ag-specific $CD8^+$ T cells which undergo proliferation under the influence of costimulation and APC-derived IL-12. The proximal daughter upregulates the T box expressed in T cells transcription factor (T-BET) and Blimp-1 and develops into a short-lived effector cell (SLEC) while the distal daughter becomes a long-lived memory CD8 T (T_M) cell. **Region 5:** during CD8 T cell expansion some effector cells do not down regulate CD127, and under the influence of IL-7 differentiate into memory potential effector cells (MPECS). **Region 6:** the sporozoite migrates through the Kupffer cell (KC) and several hepatocytes to lodge in a terminal hepatocyte within a parasitophorous vacuole. The CD8 effector T cells leave the lymph node and migrate to the space of Disse or into the sinusoid of the liver. They recognize parasite Ag plus class I on the surface of the infected hepatocyte and elaborate IFN-γ and perforin/granzyme (P/G) to kill the parasite. **Region 7:** as a result of cell traversal or of protein shedding, KC and other APC also process and present parasite Ags to activate CD4 and CD8 T cells in the sinusoid of the liver. These cells produce TNF-α which can also act at a distance to kill the parasite. The cytokine milieu of the liver might also promote the activation of regulatory T cells (T_{reg}), and these could secrete IL-10 to suppress the activation of effector T cells.

Key research questions

RTS,S vaccine trials have shown that both Abs and CD4 T cells specific for the CS protein confer sterile immunity to about 50% of malaria-naïve volunteers and partial immunity to children in endemic areas. RAS sporozoite vaccines also confer sterile immunity and, in the mouse model, CD8 T cells specific for currently unidentified liver stage Ags appear to be the key mediators of protection. Most recently, data from a fowlpox prime and MVA boost regimen (using the ME-TRAP antigen) suggest that T lymphocytes can provide sterile immunity in humans in the absence of significant antibody responses [65]. Hence, there appear to be multiple immune responses that can be harnessed to achieve pre-erythrocytic stage immunity. Key outstanding questions regarding the induction and maintenance of protective immunity to pre-erythrocytic stage antigens are listed in Table 1.

Conclusions and future directions

Long-term maintenance of high titers of sporozoite surface-Ag-specific Abs will be an important component of the protective immunity induced by vaccines, probably depending upon the generation of long-lived PCs. In general, stronger primary Ab responses correlate with stronger and more long-lived booster responses, and therefore adjuvants and vaccine platforms that favor strong primary responses could contribute to the longevity of the Ab response. Longer times between prime and boost immunizations also favor long-lived Ab production [66]. Enhancing the expression of the transcription repressor Blimp-1, which promotes the differentiation of B cells into long-lived PC [67], might be achieved by activating IL-21-secreting T_{FH} cells, and this could also favor durable Ab production.

An effective malaria vaccine should also induce strong and long-lasting pre-erythrocytic stage T cell immunity. The identification of the most protective pre-erythrocytic stage Ags is a primary objective. It will be necessary to establish whether intra-hepatic T_E cells or T_{CM} cells are the main contributors to protracted T cell immunity and to confirm whether the preservation of T_E cells requires a persistent Ag depot. It follows that adjuvants that create an Ag depot or a recombinant virus generating persistent latent infection [68] might help to maintain multi-functional T_{EM} cells in the peripheral tissue. The highly successful yellow fever vaccine targets four different TLRs located on distinct sub-populations of DCs, each with its own unique booster-effect on the immune system [69]. Hence, a combination of malaria Ags with multiple component adjuvants that target different TLRs could lead to a T_{H1}/T_{H2} balance favoring long-lived Ab production and long-lived T cell memory. Other adjuvants such as Iscomatrix (cage-like structures consisting of saponin, phospholipid and cholesterol) could also be considered, to enhance Ab as well as CD4 and CD8 T cell responses in humans [70].

Alternatively, vaccine platforms with intrinsic immunostimulatory properties could be employed to achieve a similar broad activation of humoral and cellular immunity. Combining RTS,S with a viral vector expressing either CS protein or other appropriate sporozoite or liver-stage Ags in a prime and boost regimen could induce both strong Ab and CD4 and CD8 T cell responses leading to more durable protection [71]. In support, a study in macaques demonstrated that two doses of RTS,S following an adenovirus 35 CS-protein priming immunization retained the RTS,S-induced Ab response while promoting a markedly higher CD4 T-cell response that remained elevated for about 2 months [72].

Finally, a subunit vaccine will need to be immunogenic in humans, who generally exhibit a high degree of genetic

Table 1. Outstanding issues in malaria vaccine development

Ab-mediated immunity	Refs
Identify mechanisms that maintain high titers of CS-protein-specific Abs for a prolonged period of time	[33–36,66,67,69]
Determine the role of Abs specific for non-CS-protein sporozoite Ags	[16,73,74]
Establish the contribution to protection of Ab responses targeting malaria proteins expressed on the surface of infected hepatocytes	[75]
Identify mechanisms to induce production of high-avidity Abs	[38]
T cell-mediated immunity	
Identify the key sporozoite and liver-stage Ags that stimulate protective CD4 and CD8 T cell-mediated immunity	[23–25,64]
Determine if there is a requirement for persisting CD4/CD8 T_E cells or CD4/CD8 T_{CM} cells (or both) for long-term protection	[50,51,54,58,59,61,76]
Establish whether there is a need for chronic stimulation from a persisting Ag depot to maintain long-lived intra-hepatic CD4/CD8 T_E cells	[36,49,60,61]
Characterize the relative contribution of multi-functional CD4/CD8 T cells versus unifunctional CD4/CD8 T cells in conferring protection	[27,41,42]
Identify the primary site (liver or lymph node draining the liver) of pre-erythrocytic stage Ag-specific CD4/CD8 T cell activation	[15,51,54,69]
Determine whether CD4/CD8 T cells induced by subcutaneous or intramuscular immunization with subunit vaccines migrate to the liver	[51]
Identify a cross-presentation pathway that can be targeted by exogenous Ag to activate CD8 T cells	[54,77,78]
Establish whether CD4 T_{FH} cells promote the differentiation of pre-erythrocytic stage-specific B_M cells and long-lived PC cells	[33]
Determine whether regulatory T cells inhibit responses to pre-erythrocytic stage Ags	[7,44–47,56]
Elucidate to what extent host and parasite genetic diversity affect CD4/CD8 T-cell responses to pre-erythrocytic stage Ags	[19,21,79]

Abbreviations: CS, circumsporozoite; Ab, antibody; Ag, antigen; T_E, effector T cell; T_{CM}, central memory T cell; T_{FH}, follicular helper T cell; B_M, memory B cell; PC, plasma cell.

diversity. One approach to overcoming genetic restriction would be to employ a subunit vaccine containing epitopes from multiple antigens, including universal epitopes that bind to multiple HLA types. In addition, it might be possible to choose epitopes that are restricted by HLA supertypes.

In summary, a successful pre-erythrocytic stage malaria vaccine will have to induce a durable antibody response against the sporozoite and a protracted CD4 and CD8 protective T cell response against the liver stages of the parasite. These goals should be achievable using the appropriate immunization regimens in conjunction with newly developed adjuvants and vaccine delivery vehicles. A malaria vaccine will also have to address human genetic diversity, possibly by including universal T cell epitopes, as well as the genetic diversity in the parasite itself. Clearly, we are moving closer to the development of a successful malaria vaccine, but much work remains to be done.

Disclaimer

The study protocols for the clinical trials discussed in this manuscript that were performed by the US Navy or US Army were approved by the Naval Medical Research Center or Walter Reed Army Institute of Research Institutional Review Boards, respectively, in compliance with all applicable Federal regulations governing the protection of human subjects. The animal experiments reported herein that were conducted by the US Navy or US Army were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the *Guide for the Care and Use of Laboratory Animals*, Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996. T.L.R. is a military service member and R.S. is a contract employee of Clinical Research Management, Hinkley, Ohio. The work of T.L.R. and R.S. was prepared as part of official government duties. Title 17 U.S.C. §105 provides that 'Copyright protection under this title is not available for any work of the United States Government.' Title 17 U.S.C. §101 defines a US Government work as a work prepared by a military service member or employee of the US Government as part of that person's official duties. The work of T.L.R. was supported by work unit number 6000.RAD1.F. A0309.

The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, the Department of the Army, the Department of Defense, or the US Government.

Acknowledgments

We gratefully acknowledge Ute Frevert of New York University for allowing us to modify and republish panel (a) of Figure 1.

References

1 WHO (2009) *World Malaria Report*, World Health Organization
2 Wells, T. and Poll, E. (2010) When is enough enough? The need for a robust pipeline of high-quality antimalarials. *Discov. Med.* 9, 389–398
3 Vekemans, J. *et al.* (2009) Development of the RTS,S/AS malaria candidate vaccine. *Vaccine* 27 (Suppl. 6), G67–71
4 Mota, M. (2001) Migration of *Plasmodium* sporozoites through cells before infection. *Science* 291, 141–144
5 Frevert, U. *et al.* (2005) Intravital observation of *Plasmodium berghei* sporozoite infection of the liver. *PLoS Biol.* 3, 1034–1046
6 Frevert, U. (2004) Sneaking in through the back entrance: the biology of malaria liver stages. *Trends Parasitol.* 20, 417–424
7 Guilbride, D. *et al.* (2010) Why functional pre-erythrocytic and bloodstage malaria vaccines fail: a meta-analysis of fully protective immunizations and novel immunological model. *PLoS ONE* 5, e10685–e10706
8 Clyde, D. *et al.* (1973) Immunization of man against sporozoite-induced *falciparum* malaria. *Am. J. Med. Sci.* 266, 169–177
9 Hoffman, S. *et al.* (2002) Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. *J. Infect. Dis.* 185, 1155–1164
10 Nussenzweig, R. *et al.* (1967) Protective immunity produced by the injection of x-irradiated sporozoites of *Plasmodium berghei*. *Nature* 216, 160–162
11 Nussenzweig, R. *et al.* (1972) Sporozoite-induced immunity in mammalian malaria. A review. *Am. J. Trop Med. Hyg.* 21, 722–728
12 Vaughan, A. *et al.* (2010) Genetically engineered, attenuated whole-cell vaccine approaches for malaria. *Hum. Vaccin.* 6, 107–113
13 Zechini, B. *et al.* (1999) *Plasmodium berghei* development in irradiated sporozoite-immunized C57BL6 mice. *Parasitology* 118, 335–338
14 Reiff, S. and Striepen, B. (2009) The gatekeeper revealed. *Nature* 459, 918–919
15 Bongfen, S. *et al.* (2007) *Plasmodium berghei*-infected primary hepatocytes process and present the circumsporozoite protein to specific CD8 T cells *in vitro*. *J. Immunol.* 178, 7054–7063
16 Kumar, K. *et al.* (2006) The circumsporozoite protein is an immunodominant protective antigen in irradiated sporozoites. *Nature* 444, 937–940
17 Stewart, M. and Vanderberg, J. (1988) Malaria sporozoites leave behind trails of circumsporozoite protein during gliding motility. *J. Protozool.* 35, 389–393
18 Singh, A. *et al.* (2007) *Plasmodium* circumsporozoite protein promotes the development of the liver stages of the parasite. *Cell* 131, 492–504
19 Sinigaglia, F. *et al.* (1988) A malaria T-cell epitope recognized in association with most mouse and human MHC class II molecules. *Nature* 336, 778–780
20 Nardin, E. and Nussenzweig, R. (1993) T cell responses to pre-erythrocytic stages of malaria: role in protection and vaccine development against pre-erythrocytic stages. *Annu. Rev. Immunol.* 11, 687–727
21 Doolan, D. *et al.* (1997) Degenerate cytotoxic T cell epitopes from *P. falciparum* restricted by multiple HLA-A and HLA-B supertype alleles. *Immunity* 7, 97–112
22 Calvo-Calle, J. *et al.* (2005) Human $CD4^+$ T cells induced by synthetic peptide malaria vaccine are comparable to cells elicited by attenuated *Plasmodium falciparum* sporozoites. *J. Immunol.* 175, 7575–7585
23 Sacci, J., Jr *et al.* (2005) Transcriptional analysis of *in vivo Plasmodium yoelii* liver stage gene expression. *Mol. Biochem. Parasitol.* 142, 177–183
24 Tarun, A. *et al.* (2008) A combined transcriptome and proteome survey of malaria parasite liver stages. *Proc. Natl. Acad. Sci. U.S.A.* 105, 305–310
25 Aguiar, J. *et al.* (2004) High-throughput generation of *P. falciparum* functional molecules by recombinational cloning. *Genome Res.* 14, 2076–2082
26 Stoute, J. *et al.* (1997) A preliminary evaluation of a recombinant circumsporozoite protein vaccine against *Plasmodium falciparum* malaria. *N. Engl. J. Med.* 336, 86–91
27 Kester, K. *et al.* (2009) Randomized, double-blind, phase 2a trial of *falciparum* malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection. *J. Infect. Dis.* 200, 337–346
28 Moorthy, V. and Ballou, R. (2009) Immunological mechanisms underlying protection mediated by RTS,S: a review of the available data. *Malaria J.* 8, 312–319
29 Potocnjak, P. *et al.* (1980) Monovalent fragments (Fab) of monoclonal antibodies to a sporozoite surface antigen (Pb44) protect mice against malarial infection. *J. Exp. Med.* 151, 1504–1513
30 Charoenvit, Y. *et al.* (1991) Monoclonal, but not polyclonal, antibodies protect against *Plasmodium yoelii* sporozoites. *J. Immunol.* 146, 1020–1025
31 Hollingdale, M. *et al.* (1984) Inhibition of entry of *Plasmodium falciparum* and *P. vivax* sporozoites into cultured cells; an *in vitro* assay of protective antibodies. *J. Immunol.* 132, 909–913

32 Kebaier, C. *et al.* (2009) Kinetics of mosquito-injected *Plasmodium* sporozoites in mice: fewer sporozoites are injected into sporozoites-immunized mice. *PLoS Pathogens* 5, e1000399
33 McHeyzer-Williams, L. *et al.* (2009) Follicular helper T cells as cognate regulators of B cell immunity. *Curr. Opin. Immunol.* 21, 266–273
34 Hatakeyama, S. *et al.* (2005) Persisting humoral antiviral immunity within the Japanese population after the discontinuation in 1976 of routine smallpox vaccinations. *Clin. Diagn. Lab. Immunol.* 12, 520–524
35 Poland, J. *et al.* (1981) Persistence of neutralizing antibody 30-35 years after immunization with 17D yellow fever vaccine. *Bull. World Health Organ.* 59, 895–900
36 Tew, J. *et al.* (1997) Follicular dendritic cells and presentation of antigen and costimulatory signals to B cells. *Immunol. Rev.* 156, 39–52
37 Alonso, P. *et al.* (2004) Efficacy of the RTS,S/AS02A vaccine against *Plasmodium falciparum* infection and disease in young African children: randomised controlled trial. *Lancet* 364, 1411–1420
38 Feng, J. *et al.* (2009) Antibody quantity versus quality after influenza vaccination. *Vaccine* 27, 6358–6362
39 Rénia, L. *et al.* (1991) *In vitro* activity of $CD4^+$ and $CD8^+$ T lymphocytes from mice immunized with a synthetic malaria peptide. *Proc. Natl. Acad. Sci. U.S.A.* 88, 7963–7967
40 Ferreira, A. *et al.* (1986) Inhibition of development of exoerythrocytic forms of malaria parasites by gamma-interferon. *Science* 232, 881–884
41 Darrah, P. *et al.* (2007) Multifunctional TH1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. *Nat. Med.* 13, 843–850
42 Kannanganat, S. *et al.* (2007) Multiple-cytokine-producing antiviral CD4 T cells are functionally superior to single-cytokine-producing cells. *J. Virol.* 81, 8468–8476
43 Seder, R. *et al.* (2008) T-cell quality in memory and protection: implications for vaccine design. *Nat. Rev. Immunol.* 8, 247–258
44 Curotto de Lafaille, M. and Lafaille, J. (2009) Natural and adaptive $foxp3^+$ regulatory T cells: more of the same or a division of labor? *Immunity* 30, 626–635
45 Mendez, S. *et al.* (2004) Role for $CD4^+$ $CD25^+$ regulatory T cells in reactivation of persistent leishmaniasis and control of concomitant immunity. *J. Exp. Med.* 200, 201–210
46 Casares, S. and Richie, T. (2009) Immune evasion by malaria parasites: a challenge for vaccine development. *Curr. Opin. Immunol.* 21, 321–330
47 Todryk, S. *et al.* (2008) Correlation of memory T cell responses against TRAP with protection from clinical malaria, and CD4 CD25 high T cells with susceptibility in Kenyans. *PLoS ONE* 3, e2027–e2039
48 Tarun, A. *et al.* (2007) Protracted sterile protection with *Plasmodium yoelii* pre-erythrocytic genetically attenuated parasite malaria vaccines is independent of significant liver-stage persistence and is mediated by $CD8^+$ T cells. *J. Infect. Dis.* 196, 608–616
49 Trimnell, A. *et al.* (2009) Genetically attenuated parasite vaccines induce contact-dependent $CD8^+$ T cell killing of *Plasmodium yoelii* liver stage-infected hepatocytes. *J. Immunol.* 183, 5870–5878
50 Malik, A. *et al.* (1991) Human cytotoxic T lymphocytes against the *Plasmodium falciparum* circumsporozoite protein. *Proc. Natl. Acad. Sci. U.S.A.* 88, 3300–3304
51 Chakravarty, S. *et al.* (2007) $CD8^+$ T lymphocytes protective against malaria liver stages are primed in skin-draining lymph nodes. *Nat. Med.* 13, 1035–1041
52 James, E. (2005) Apoptosis: key to the attenuated malaria vaccine? *J. Infect. Dis.* 191, 1573–1575
53 van Dijk, M. *et al.* (2005) Genetically attenuated, P36p-deficient malarial sporozoites induce protective immunity and apoptosis of infected liver cells. *Proc. Natl. Acad. Sci. U.S.A.* 102, 12194–12199
54 Jobe, O. *et al.* (2009) Immunization with radiation-attenuated *Plasmodium berghei* sporozoites induces liver $cCD8alpha^+DC$ that activate $CD8^+$ T cells against liver-stage malaria. *PLoS ONE* 4, e5075
55 Rénia, L. *et al.* (2006) Do apoptotic *Plasmodium*-infected hepatocytes initiate protective immune responses? *J. Infect. Dis.* 193, 163–164
56 Sauerwein, R. *et al.* (2010) Empowering malaria vaccination by drug administration. *Curr. Opin. Immunol.* 22, 367–373
57 Accapezzato, D. *et al.* (2005) Chloroquine enhances human CD8 T cell responses against soluble antigens *in vivo*. *J. Exp. Med.* 202, 817–828
58 Berenzon, D. *et al.* (2003) Protracted protection to *Plasmodium berghei* malaria is linked to functionally and phenotypically heterogeneous liver memory $CD8^+$ T cells. *J. Immunol.* 171, 2024–2034
59 Jobe, O. *et al.* (2007) Genetically attenuated *Plasmodium berghei* liver stages induce sterile protracted protection that is mediated by major histocompatibility complex class I-dependent interferon-γ-producing $CD8^+$ T cells. *J. Infect. Dis.* 196, 599–607
60 Scheller, L. and Azad, A. (1995) Maintenance of protective immunity against malaria by persistent hepatic parasites derived from irradiated sporozoites. *Proc. Natl. Acad. Sci. U.S.A.* 92, 4066–4068
61 Mueller, A. *et al.* (2007) Genetically attenuated *Plasmodium berghei* liver stages persist and elicit sterile protection primarily via CD8 T cells. *Am. J. Pathol.* 171, 107–115
62 Keating, S. *et al.* (2005) Durable human memory T cells quantifiable by cultured enzyme-linked immunospot assays are induced by heterologous prime boost immunization and correlate with protection against malaria. *J. Immunol.* 175, 5675–5680
63 Todryk, S. *et al.* (2009) The relationship between human effector and memory T cells measured by *ex vivo* and cultured ELISPOT following recent and distal priming. *Immunology* 128, 83–91
64 Doolan, D. *et al.* (2003) Identification of *Plasmodium falciparum* antigens by antigenic analysis of genomic and proteomic data. *Proc. Natl. Acad. Sci. U.S.A.* 100, 9952–9957
65 Webster, D. *et al.* (2005) Enhanced T cell-mediated protection against malaria in human challenges by using the recombinant poxviruses FP9 and modified vaccinia virus Ankara. *Proc. Natl. Acad. Sci. U.S.A.* 102, 4836–4841
66 Siegrist, C. (2004) Vaccine Immunology. In *Vaccines* (Plotkin, S. *et al.*, eds), pp. 17–36, Elsevier
67 Shapiro-Shelef, M. *et al.* (2003) Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity* 19, 607–620
68 Hansen, S. *et al.* (2009) Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. *Nat. Med.* 15, 293–299
69 Querec, T. *et al.* (2006) Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2,7,8 and 9 to stimulate polyvalent immunity. *J. Exp. Med.* 203, 413–424
70 Reed, S. *et al.* (2008) New horizons in adjuvants for vaccine development. *Trends Immunol.* 30, 23–32
71 Hutchings, C. *et al.* (2007) Combination of protein and viral vaccines induces potent cellular and humoral immune responses and enhanced protection from murine malaria challenge. *Infect. Immun.* 75, 5819–5826
72 Stewart, V. *et al.* (2007) Priming with an adenovirus 35-circumsporozoite protein (CS) vaccine followed by RTS,S/AS01B boosting significantly improves immunogenicity to *Plasmodium falciparum* CS compared to that with either malaria vaccine alone. *Infect. Immun.* 75, 2283–2290
73 Mauduit, M. *et al.* (2009) A role for immune responses against non-CS components in the cross-species protection induced by immunization with irradiated malaria sporozoites. *PLoS ONE* 4, e7717
74 Rogers, W. *et al.* (1992) Characterization of *Plasmodium falciparum* sporozoite surface protein 2. *Proc. Natl. Acad. Sci. U.S.A.* 89, 9176–9180
75 Mazier, D. *et al.* (1990) Hepatic phase of malaria is the target of cellular mechanisms induced by the previous and the subsequent stages. A crucial role for liver nonparenchymal cells. *Immunol. Lett.* 25, 65–70
76 Frevert, U. and Nardin, E. (2008) Cellular effector mechanisms against *plasmodium* liver stages. *Cell. Microbiol.* 10, 1956–1967
77 Bonifaz, L. *et al.* (2004) *In vivo* targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. *J. Exp. Med.* 199, 815–824
78 Wille-Reece, U. *et al.* (2005) Immunization with HIV-1 Gag protein conjugated to a TLR7/8 agonist results in the generation of HIV-1 Gag-specific Th1 and $CD8^+$ T cell responses. *J. Immunol.* 174, 7676–7683
79 Plebanski, M. *et al.* (1999) Interleukin 10-mediated immunosuppression by a variant CD4 T cell epitope of *Plasmodium falciparum*. *Immunity* 10, 651–660